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DOUBLE LIPOSOMES MEDIATED DUAL THERAPY OF AIDS RELATED OPPORTUNISTIC FUNGAL INFECTIONS AND AIDS

Babu Ram Garg*[1], Arun Kumar[2], Minakshi Garg[3], Jayamanti Pandit[4] and Narendra K. Jain[5]

Himachal Institute of Pharmacy [1], Poanta Sahib, Distt. Sirmour (H. P.), India
BPS Mahila Vishwavidyalaya Khanpur Kalan, Sonipat, Haryana, India
Pharmaceutics Research Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour University, Sagar, (M. P.), India
Pharmaceutics Research Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour University, Sagar, (M. P.), India
Pharmaceutics Research Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour University, Sagar, (M. P.), India

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Correspondence to Author:

Babu Ram Garg

Principal, Himachal Institute of Pharmacy, Poanta Sahib, Distt. Sirmour (H. P.), India

E- mail: brgarg2005@yahoo.co.in

ABSTRACT

To deliver zidovudine (AZT) to asialoglycoprotein receptors, drug-containing galactosylated liposomes were prepared. Simultaneously, miconazole nitrate (MCZ) was entrapped in the outer liposomal bilayer of double liposomes for the treatment of opportunistic fungal infections. Galactosylated liposomes were entrapped in double liposomes. The galactose binding *Ricinus communis* lectin was used for the determination of in vitro ligand binding capacity. Percent cumulative drug release from double liposomes with inner uncoated liposomes (DLMA) and inner galactosylated liposomes (Gal-DLMA) were compared. Cellular drug uptake studies were performed. In vivo antifungal activity, plasma distribution and hepatic localization study were performed in albino rats. Percent cumulative drug release of AZT from inner liposomes was significantly higher with DLMA as compared to Gal-DLMA. MCZ uptake was 4.7 times greater after encapsulation in liposomes irrespective of inner liposome coating and maximum AZT uptake (7.9 fold enhancement) was observed from double bilayer galactosylated liposomes as compared to free drug. MCZ encapsulated in double liposomes was able to reduce CFU (colony forming units) values to a significant extent in various tissues. Gal-DLMA remains in the body for longer period of time. In case of Gal-DLMA administration significant amount of AZT was recovered from parenchymal cells of liver as compared to non-parenchymal cells. Galactosylated lipid substances allowed liver specific uptake of AZT at enhanced parenchymal: non-parenchymal selectivity ratios and at the same time could deliver MCZ for treatment of fungal infections. Galactosylated liposomes further entrapped in liposomes hold much promise in dual drug delivery.

INTRODUCTION: The rates of opportunistic fungal infections in acquired immuno deficiency syndrome (AIDS) can range from 60 percent to 90 percent for mucosal candidiasis, from 6 percent to 30 percent for invasive mycoses (such as cryptococcosis, histoplasmosis and coccidioi-mycosis) (www.niaid.nih.gov). AIDS patients may also be on treatment for opportunistic infections. Opportunistic infections caused by certain fungi, in particular problematic *Candida albicans*, have increased recently and become a public concern. Patients with compromised immune systems, e.g. patients receiving organ transplants and cancer chemotherapy, or those infected by human immunodeficiency virus (HIV), are particularly prone to such infections.

Azole derivatives, which inhibit fungal ergosterol biosynthesis by blockade of the cytochrome P-450 reaction involved in 14-α demethylation, are the most commonly used agents. Oral candidiasis is the most common opportunistic fungal infection in persons suffering from AIDS. Candida is also deposited in lungs, liver, spleen and kidney [1].

Miconazole nitrate (MCZ) is a widely used antifungal agent, but its use in topical formulations is not efficacious because deep-seated fungal infections are difficult to treat with conventional topical formulations. Oral formulations of MCZ are associated with various adverse effects such as nausea, vomiting and diarrhea [2]. Sustained release parenteral formulation (liposomes) will not only be efficacious but also reduce the toxicity [3-4]. Zidovudine (AZT) is one of the most important drugs in AIDS-therapy [5].

Oral dosage (200mg/every 4 h) often makes blood levels exceed toxic levels, causing severe adverse effects such as granulocytopenia and anemia. Hence, the controlled delivery of AZT through non-oral pathways such as the parenteral route can be a useful way to circumvent these problems, if sufficient amount of AZT can be delivered [6]. Liposomal encapsulation of AZT can positively alter the pharmacokinetic profile of this antiviral agent by increasing its half-life in blood. Double liposome based galactosylated liposomal drug delivery system may be ideal in the case of MCZ and AZT in that, it may improve availability by means of a controlled-release pattern, can advance the treatment of opportunistic fungal infections and it can alleviate drug toxicity by delivering the drug directly to the infected cells in a passive manner, respectively.

Double liposomes are better than a mixture of two populations of liposomes; one loaded with AZT and the other with MCZ as the PEGylated outer liposomes. The outer liposomes provide long circulatory effect by preventing its opsonization and subsequent degradation by the macrophages, thereby prolonging the residence time of the drug within the body while the inner galactosylated liposomes diffuse out of the outer liposomes and are specifically taken up by the galactose /lectin receptors of the mononuclear phagocytic cell and the hepatocytes. Asialoglycoprotein receptor specific for galactose is present on parenchymal cells of hepatocytes. Galactosylated liposomes are selectively removed from circulation by Kupffer cells.

Kupffer cells carry on the cell surface a lectin-like receptor with specificity for D-galactose residues. Human monocytes and dendritic cells, but not cells of the B lineage, bear galactose receptor [7-9]. These galactose receptor-bearing cells are the reservoirs of HIV-I. The present investigation was undertaken to deliver MCZ in a controlled manner and direct AZT to HIV infected cells having galactose specific receptor. This goal has been achieved by double liposomes carrying galactosylated liposomes in their core. Cellular uptake, *in vivo* antifungal activity, hepatic

localization and plasma distribution of free MCZ and AZT, double liposomes (uncoated inner liposomes) and double liposomes galactosylated inner liposomal AZT system) carrying MCZ and AZT were studied *in vivo* following a bolus intravenous injection in rats.

MATERIALS AND METHODS:

Materials: Zidovudine (AZT) and Miconazole nitrate (MCZ) were received as gift sample from M/s Hetero Drugs, Hyderabad, India and Zyg Pharmaceuticals, Indore, India, respectively. Egg phosphatidylcholine, cholesterol (CH), phosphatidyl -ethanolamine (PE), galactose, sephadex G-50, Ricinus communis lectin, dimethyl sulfoxide (DMSO), Ficoll- Hypaque, Dulbecco's Modified Eagle's Medium high glucose (DME-HG), L-glutamine, penicillin, streptomycin, heat-inactivated fetal bovine serum, human AB serum and Triton X-100 were purchased from Sigma Chemicals Co. (USA).

1, 2- Distearoyl- sn- glycero- 3-phospho-ethanolamine- N - polyethylene glycerol (PEG_{350}-DSPE) was purchased from Avanti Polar Lipids (Alabaster, AL). Hank's balanced salt solution was obtained from Himedia, Mumbai, India. Dimethoxyethane, dicyclohexyl carbodiimide (DCC), 3- thiopropionic acid, boron trifluoro-etherate, dichloromethane (DCM) and N- hydroxy succinimide (NHS) were procured from Spectrochem, Mumbai, India. Sabouraud dextrose agar was obtained from Merck, Mumbai, India. *Candida albicans* was obtained from Institute of Microbial Technology, Chandigarh, India (MTCC*1637).

Synthesis of Thiol Functionalized β-D-Galactosyl Residue: Galactosylated phosphatidylethanolamine was synthesized following the method described earlier [10] with little modification in our laboratory. Galactose was activated to its acetylated derivative containing an activated ester group, the reactive end group for conjugation. Briefly, 19 mL of acetic anhydride cooled down to a temperature 4°C and 0.1mL of 60% perchloric acid was added to it drop wise with stirring. The reaction mixture was then allowed to obtain the room temperature. Six grams of galactose was added to this mixture with stirring and the temperature of the mixture was maintained at 35°C. Reaction mixture was allowed to stand for 24 h at 25°C. The brown colored solution thus obtained was transferred drop wise to 25mL distilled water with stirring. The insoluble brown colored sticky galactose pentaacetate got separated. Galactose pentaacetate (m.p. - 85°C) so obtained was dried in vacuum desiccator.

Two grams of galactose pentaacetate was dissolved in 25mL dichloromethane. To the resulting solution 3-thiopropionic acid (1.6mL) and boron trifluoroetherate (0.9mL) were added. The mixture was stirred overnight at 25°C, before being diluted with dichloromethane (25mL) and washed with 1M HCl (4x50mL). The yellow colored mixture was evaporated and further dried in vacuum desiccator. A solution of thioglycoside synthesized in dimethoxyethane was treated with DCC (600mg) and NHS (315mg) at 0-10°C for 20 h with stirring.

The precipitate was filtered off and the solvent was removed from filtrate under vacuum. The residue obtained was dissolved in 10mL chloroform, filtered and concentrated. This concentrated reaction mixture was poured in cyclohexane (25mL) to precipitate the product. The precipitation step was repeated. The solvent was decanted.

The viscous oily product so obtained was dried to get white product (N-Hydroxy succinimidoyl-3- (2, 3, 4, 6- tetra- O- acetyl- β- D-galactopyranosylthio) - propionate). Phosphatidyl ethanolamine (20mg) was dissolved in 10mL solution of DCM and chloroform (50: 50v/v). The white product obtained above was added to this

solution. The mixture was stirred at 25°C 48 h. After dilution with DCM (25mL) the solution was washed with saturated aqueous sodium carbonate (3 x 50mL) and dried over sodium sulfate. The solution was concentrated under vacuum to yield white product. The product obtained was insoluble in water.

The deacetylation was carried out by Zemplen reaction followed by treatment with an aqueous sodium hydroxide solution [11]. The galactosylated phosphatidyl- ethanolamine (Gal-PE) so formed was confirmed by IR spectrum. The FTIR spectrum was recorded on FTIR multiscope spectrophotometer (Perkin Elmer, Buckingham-shire, UK) equipped with spectrum v3.02 software, by a conventional KBr pellet method.

Preparation of Double Liposome: In the present investigation, inner liposomes were prepared using film hydration [12]. Briefly, lipids (Egg phosphatidyl-choline, CH, Gal-PE) in different ratios were dissolved in 5mL chloroform (**Table 1**). The solvent was removed completely by rotary evaporation (Rotary evaporator, York Scientific Ltd., India) under vacuum above the transition temperature of lipid. The lipid film was further dried for 1 h under vacuum. The thin, uniform lipid film thus produced was hydrated with 1mL of aqueous phase, i.e. phosphate buffer saline (PBS, pH- 7.4) containing 250μg/mL AZT for 1 h at room temperature with continuous rotation at 60-rev min^{-1}.

The resulting vesicles were allowed to swell for 2 h at room temperature to produce large multilamellar vesicles. A laboratory homogenizer (EmulsiFlex-C5, Avestin, Ottawa, ON, Canada) was used to reduce the particle size of the liposomes. Multiple passes (5k-10k psi) were often needed to obtain the desired particle size (~100nm). Liposomes were then extruded through polycarbonate membranes of 200 nm pore size. Entrapment efficiency was determined after separation of unentrapped drug by sephadex G-50 minicolumn using centrifugation technique [12-13].

The amount of drug entrapped in the vesicles was then determined by disrupting the vesicles using 0.1% Triton X-100 and the liberated drug was determined using UV spectrophotometer (Shimadzu 1601) at 266 nm. Differential scanning calorimetry (DSC) was performed with a Perkin-Elmer differential calorimeter (DSC7, Perkin-Elmer, Nortwalk, Conecticut, USA). Liposomes with and without AZT were placed in conventional aluminium pan and a scan speed of 2°C/min was employed. The weight of each sample was 15±2mg.

TABLE 1: OPTIMIZATION OF LIPID RATIO ON THE BASIS OF CHARACTERIZATION PARAMETERS FOR INNER LIPOSOMES

Formulation code	Molar lipid ratios (Egg phosphatidylcholine: CH: Gal PE)	Particle size (nm)*	Particle shape	% Entrapment efficiency*
L1	9:0.5:0.5	176.25 ± 1.1.9	Nearly spherical	29.02 ± 0.45
L2	7:2.5:0.5	130.45 ± 1.24	Spherical	42.38 ± 2.58
L3	6:3.5:0.5	182.02 ± 5.25	Nearly spherical	18.25 ± 1.25
L4	5:4.5:0.5	190.70 ± 3.2	Deformed	15.28 ± 1.14

L1 to L4 are the formulation codes for inner liposomes. CH and Gal-PE are the abbreviations for cholesterol and galactosylated phosphatidyl ethanolamine, respectively. *All values are expressed as mean ± S.D. (n = 6)

Egg Phosphatidylcholine, PEG_{350}-DSPE and CH were dissolved in chloroform in different molar ratios (**Table 2**). Miconazole nitrate (6.25mg) was dissolved in minimum quantity of absolute alcohol. The two solutions were mixed. The resultant solution was transferred on to glass beads (25g) contained in a round bottom flask. The solvents were evaporated at room temperature to form a thin lipid film containing MCZ over glass beads. This film was hydrated with the 1.5mL dispersion of

inner liposomes, with gentle shaking for 10 min [14-15]. Vesicle size of inner and finally formed double liposomes was evaluated by dynamic light scattering using Coulter N4 MD Submicron Particle Size Analyzer (Coulter Electronics, Hialeah, FL). Double Liposomes were visualized using optical microscope (Leica, Germany) and transmission electron microscope (Philips, Japan) with an accelerating voltage of 120 kv. DSC measurements of double liposomes with and without MCZ were done and a scan speed of 2°C/min was employed. The weight of each sample was 15±2mg.

TABLE 2: OPTIMIZATION OF LIPID RATIO ON THE BASIS OF CHARACTERIZATION PARAMETERS FOR OUTER LIPOSOMES

Formulation code	Molar lipid ratios (Egg phosphatidylcholine: CH: PEG_{2000}-DSPE)	Particle size (μm)*	Particle shape	% Entrapment efficiency*
DL1	9:1:0.09	2.51 ± 0.21	Nearly spherical	20.15 ± 2.41
DL2	7:3:0.09	1.20 ± 0.01	Spherical	31.62 ± 1.52
DL3	6:4:0.09	4.24 ± 0.76	Nearly spherical	15.11 ± 1.77
DL4	5:5:0.09	5.7 ± 1.02	Deformed	12.24 ± 0.78

DL1 to DL4 are the formulation codes for double liposomes. CH and PEG_{2000}-DSPE are the abbreviations for cholesterol and 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene glycerol, respectively. *All values are expressed as mean ± S.D. (n = 6)

***In Vitro* Specificity of Galactosylated Liposomes:** The Gal-PE liposomal system was assessed for in vitro ligand-specific activity by *Ricinus communis* lectin agglutination assay [16]. 1mL of original double liposomal formulations (with inner uncoated and galactosylated liposomes) were incubated with increasing concentrations (5, 10, 20, 30, 50 and 60 μg/mL) of *Ricinus communis* agglutinin, in a cuvette containing 1mL PBS (pH- 7.4) at 25°C. Time dependent (0-160 min) increase in turbidity at 360 nm was monitored spectrophotometrically (Shimadzu 1601 UV spectrophotometer, Japan).
The same study was repeated with galactose coated inner liposomes of equivalent concentration (5, 10, 20, 30, 50 and 60 μg/mL).

Drug Release Studies: The drug release from liposomes was studied by dialysis cell membrane method. 2mL each of double liposomes with inner uncoated liposomes (comprised of PC: CH:PE) and double liposomes with galactosylated liposomal formulations were dialysed against 100mL of PBS (pH 7.4) and the solution was continuously stirred using magnetic stirrer at 37±1°C. After appropriate time intervals (1 h), 1mL of sample was withdrawn and analyzed for drug content. Equal volume of fresh media was added to replace the withdrawn sample. MCZ and AZT release were measured at 235 and 266nm, respectively using UV spectrophotometer (Shimadzu 1601). Every experiment was performed in triplicate.

Cellular Drug Uptake Studies: Whole human blood was collected in HiAnticlot blood collection vials (Himedia Labs, India). The blood was diluted twice with balanced salt solution and allowed to stand for 1 h on Ficoll-Hypaque (diluted blood: Ficoll-Hypaque, 3:1). The sample was centrifuged at 400 X g; 25°C for 30 min. Mononuclear lymphocytes and monocytes were recovered as a white band at the ficoll plasma interface. These cells at the interface were aspirated. Cells were washed thrice with saline at 600-700 X g for 10 min.

Supernatant was removed. The pellet obtained was washed twice with RPMI at 100 g for 10 min. Supernatant was discarded and the pellet was suspended in DME-HG. The cells were plated in DME-HG supplemented with L-glutamine (4mM), penicillin (100 units/mL) and streptomycin (100μg/mL). Cells were allowed to adhere overnight, followed by removal of non-adherent cells by gentle washing, and the medium was replaced with DME-HG containing 20% heat-inactivated fetal bovine serum, 10% human AB

serum and with L-glutamine (4mM), penicillin (100 units/mL) and streptomycin (100μg/mL). The cells were allowed to differentiate for 6 days. Subsequent cultures were carried out in DME-HG with 20 % heat-inactivated fetal bovine serum and L-glutamine, penicillin and streptomycin as above in an incubator with 5% CO_2 at 37°C.

About 100μl of the monocytic cell suspension, corresponding to a seeding density of 1 x 10^6 cells/mL, was transferred to 48 well culture plates. Ten microlitres of plain drug solution (2.5 mg MCZ + 0.15 mg AZT in 1.5 ml 0.4 % DMSO solution), double liposomes with uncoated inner liposomes (DLMA) and double liposomes with galactosylated inner liposomes (Gal-DLMA) having equivalent amount of drugs were then added to 6 wells of I, II and III columns, respectively. DMSO (0.4 %), double liposomes with uncoated inner liposomes (without drug) and double liposomes with galactosylated inner liposomes (without drug) were added to the remaining 6 wells of I, II and III columns, respectively; which served as reference to the loaded formulations.

Ten microlitres of drug unloaded double liposomes with galactosylated inner liposomes were added to 6 wells of column IV to study the inhibitory effect of these liposomes on the uptake of zidovudine and miconazole nitrate containing galactosylated liposome (Gal-DLMA). The plates were incubated in a controlled environment at a temperature of 37±1°C for a period of 48 h. 10 microlitres of Gal-DLMA was added to 6 wells of column IV and incubated for further 48 h at 37±1°C. During first incubation of the plates, at appropriate time points of 0, 1, 4, 8, 20 and 48 h the cell suspension from each well of I, II, III columns and during second incubation of plate the cell suspension from each well IV column was transferred to polycarbonate filters (pore size 0.45 m), the wells of the cell culture plates rinsed with 1 mL PBS (pH 7.4) and the washings subsequently transferred to the polycarbonate filters. The cells were separated from the medium, in the form of a pellet by centrifuging the filters at 4000 rpm for 15 min. About 0.5mL of Triton X-100 was added to the pellet to rupture the cells and the mixture incubated at 25°C for 5-6 h. Drugs (MCZ and AZT) uptake was determined by HPLC method.

The AZT and MCZ were assayed using a high-performance liquid chromatography system (Jasco, model 821-FP, Tokyo, Japan). The mobile phase for AZT assay consisted of 0.1% acetonitrile and acetic acid solution (85:15). Sample was passed at a flow-rate of 1mL/min by LC10 AT, pump on a 5μ - Luna C18 column (Phenomenex, USA) with UV detection at 266nm using photo diode array detector (SPD-M10A, Shimadzu, Japan) (Oh et al 1998). The mobile phase for MCZ assay comprised of 0.6 % w/v solution of ammonium acetate in a solution of acetonitrile, methanol and water (15:16:19). Sample was passed at a flow-rate of 2 mL/min with UV detection at 235 nm [17].

In vivo Antifungal Activity: Healthy male albino rats (Sprague-Dawley strain) of uniform body weight (100±20 g) with no prior drug treatment were used for the in vivo studies. The animals were fed on a commercial pellet diet (Hindustan Lever, Bangalore, India), and water ad libitum. The animals were acclimatized to laboratory hygienic conditions for 10 days before commencing the experiment.

The animals were divided into seven groups having twenty rats in each group. The animals were infected with 0.25mL *Candida albicans* cell suspension (1x10^6 cells/mL) in normal saline via the caudal vein. Free MCZ (free MCZ) solution in 0.4 % DMSO (25 mg/kg), free MCZ + AZT solution in 0.4 % DMSO (2.5mg MCZ + 0.15mg AZT/1.5mL solution), 1.5mL Gal-DLMA (2.5mg MCZ + 0.15mg AZT/1.5mL Gal-DLMA), 1.5mL DLMA (2.5mg MCZ + 0.15mg AZT/1.5mL DLMA) and 1.5mL Gal-DLA (0.15mg

AZT/1.5mL of Gal-DLA; double liposomes with galactosylated inner liposomes loaded with AZT alone), 1.5mL AZT loaded double liposomes with uncoated inner liposomes i.e. DLA (0.15mg AZT/1.5mL of DLA) were administered intravenously to groups I, II, III, IV, V and VI, respectively. Group VII received physiological saline (1mL/kg) and served as control for groups II and I while Group V and VI served as control for group III and IV, respectively.

Each group (except VII) was administered with their respective MCZ preparation on alternate days starting from the 3rd day of infection for 13 days, similarly group VI was treated with saline as control. The progress of infection and mortality of rats were monitored for 15-20 days. Number of survived rats was recorded on 20th day. Three rats from each group were killed 2 days after the last dose and the fungal load was determined, in terms of colony forming units (CFU) in lung, liver, kidney, spleen, pancreas and plasma. The organs were excised aseptically, washed with physiological saline and then homogenized in saline using tissue homogenizer (MAC Micro Tissue Homogenizer, Delhi, India). A 30-fold serial dilution was placed in Sabouraud dextrose agar plates and then counted twice for CFU after 48 h incubation at 37°C [18].

Plasma Distribution and Hepatic Localization Study: Albino rats (Sprague-Dawley strain) of either sex of uniform body weight (100±20 g) were used for the determination of tissue distribution of drugs. The rats were divided into six groups of eighteen animals each. To the first group plain drug solution (MCZ + AZT in 0.4 % DMSO; 2.5mg MCZ + 0.15mg AZT in 1.5mL solution), to the second and third groups equivalent amounts of DLMA and Gal-DLMA were administered respectively, through the caudal vein. Fourth group served as control for first group to whom 1.5mL 0.4% DMSO was administered, fifth group served as control for second group to whom plain (without drug) uncoated DL was administered and sixth group served as control for third group to whom blank double liposomes with galactosylated inner liposomes (Gal-DL) was administered. After 0.25, 0.5, 1, 2, 12 h and 24 h three animals from each group were sacrificed. Liver was excised and the capsular membrane was removed. The cells were dispersed in ice-cold Hank's HEPES buffer containing 0.1% BSA by gentle stirring. The dispersed cells were filtered through cotton mesh sieves followed by centrifugation at 50 X g for 2 min. The pellets having parenchymal (PC) cells were washed twice with hank's-HEPES buffer by centrifuging at 50 X g for 2 min. The supernatant so obtained had non-parenchymal cells (NPC).

PC and NPC were resuspended separately in ice-cold Hank's-HEPES buffer (5mL for PC and 3mL for NPC). The drug uptake was determined as mentioned earlier under cell uptake studies. Blood samples (0.5mL) were collected in 0.9% normal saline at 0.25, 0.5, 1, 2 upto 12 h). The collected blood samples were clotted and washed by vortexing with normal saline and the washings were centrifuged at 2000 rpm for 15 min. Serum was deproteinized by acetonitrile (1mL/mL of serum). The samples were centrifuged and supernatants were analyzed for drug content against similarly treated blood sample of control rats by HPLC. The area under the plasma concentration-time curve (AUC) and the area under the first moment of the concentration-time curve (AUMC) were calculated using the trapezoidal rule. The mean residence time (MRT) was calculated as AUMC/AUC.

Statistical Analysis: Statistical analysis was performed with GraphPad InStat software (version 3.00, GraphPad Software, San Diego, California USA) using oneway ANOVA followed by Tukey-Kramer multiple comparison test. Difference with $P<0.05$ was considered statistically significant.

RESULTS: Galactosylated phosphatidyl ethanolamine was characterized by IR spectrum. O-H stretch (very broad band) and strong C-O stretch of carbohydrate were obtained around 3410.2 and 1114.1 cm^{-1}, respectively. A characteristic C-H stretching and bending vibration was observed at 2943.2 cm^{-1} and 1360.3 cm^{-1}, respectively. Strong peaks at 1701.3 cm^{-1} and 1130.8 cm^{-1} confirmed the carbonyl (C=O) and C-O groups of ester present in phosphatidyl ethanolamine, respectively. Weak N-H stretch was obtained at 3150.2 cm^{-1}. Linkage of galactose with phosphatidyl ethanolamine was assured by amide linkage. The characteristic strong C=O stretch and N-H def of amide bond were obtained at 1686.4 and 1500.8 cm^{-1}, respectively. Weak stretch at 684.0 cm^{-1} confirmed C-S linkage. AZT and MCZ showed maximum absorbance at 266nm and 235nm, respectively. Variation in the lipid ratio used in the preparation of inner liposomes alters encapsulation efficiency and particle size to a significant ($P<0.05$) extent. Formulation L2 was found to have higher (42.38±2.58) entrapment efficiency and lowest particle size (130.45±1.24nm) (Table 1). Therefore, formulation L2 was chosen among others for further encapsulation in PEG_{350}-DSPE based liposomes. Entrapment efficiency of double liposomal formulation DL2 entrapping L2 was significantly ($P<0.05$) higher among other formulations (Table 2). Phase contrast microscope revealed the vesicular structure of liposomes (**Fig. 1 A-B**). Transmission electron photomicrograph clearly shows the presence of galactose coated liposomes within the PEGylated outer liposomes (**Fig. 1C**).

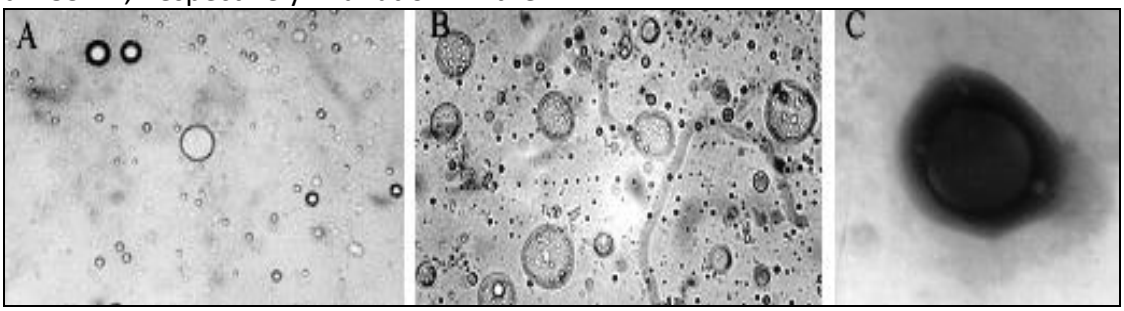


FIG. 1: (A) PHASE CONTRAST MICROSCOPY OF PLAIN LIPOSOMES (X 450); (B) PHASE CONTRAST MICROSCOPY OF DOUBLE LIPOSOMES (X 450): (C) TRANSMISSION ELECTRON PHOTOMICROGRAPH OF DOUBLE LIPOSOMES (X 11600 K; 120 KV)

DSC was used to evaluate the interactions between AZT and outer liposomal lipids. **Figure 2** shows the thermograms of liposomes. The DSC trace of Gal-PE liposomes with or without AZT showed a peak transition at 47.9±0.2°C. DSC trace of outer liposomes with or without MCZ showed a peak transition at 42.1±0.1°C. Increase in turbidity was observed after addition of varying concentrations of *Ricinus communis* lectin to double liposomal formulations (with inner Gal-PE liposomes). The rate of agglutination depends on lectin concentration.

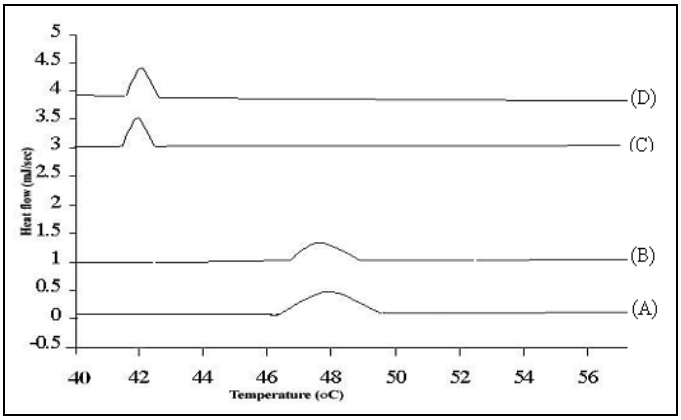


FIG. 2: DIFFERENTIAL SCANNING CALORIMETRY TRACES OF LIPOSOMES; (A) INNER GALACTOSYLATED LIPOSOMES WITHOUT AZT, (B) INNER GALACTOSYLATED LIPOSOMES WITH AZT, (C) OUTER LIPOSOMES WITHOUT MCZ (D) OUTER LIPOSOMES WITH MCZ

The turbidity changes after addition of varying concentrations of lectin to galactosylated liposomal formulation. The extent of aggregation increased as lectin concentration was increased from 5μg/mL to 30μg/mL and time from 5 min to 120 min. Above 30μg/mL lectin concentration, aggregation became constant indicating saturation of the binding sites. After 120 min no change in turbidity was observed (**Fig. 3A**). In case of double liposomal formulations (with inner PE liposomes i.e. uncoated inner liposomes) no turbidity was observed. These results are in good agreement with the findings of Haensler and Schuber [16]. In case of galactose coated inner liposomes alone immediate appearance of turbidity was observed. Here, the aggregation became constant after 20 min which might be due to immediate interaction of the galactose with the lectin (**Fig. 3B**). Comparison of the two results clearly indicates that the diffusion of galactosylated liposomes from outer liposomes accounts for the delayed attainment of saturation. This also supports the fact that the double liposomes contain galactosylated inner liposomes entrapped within the aqueous layer of the PEGylated liposomes.

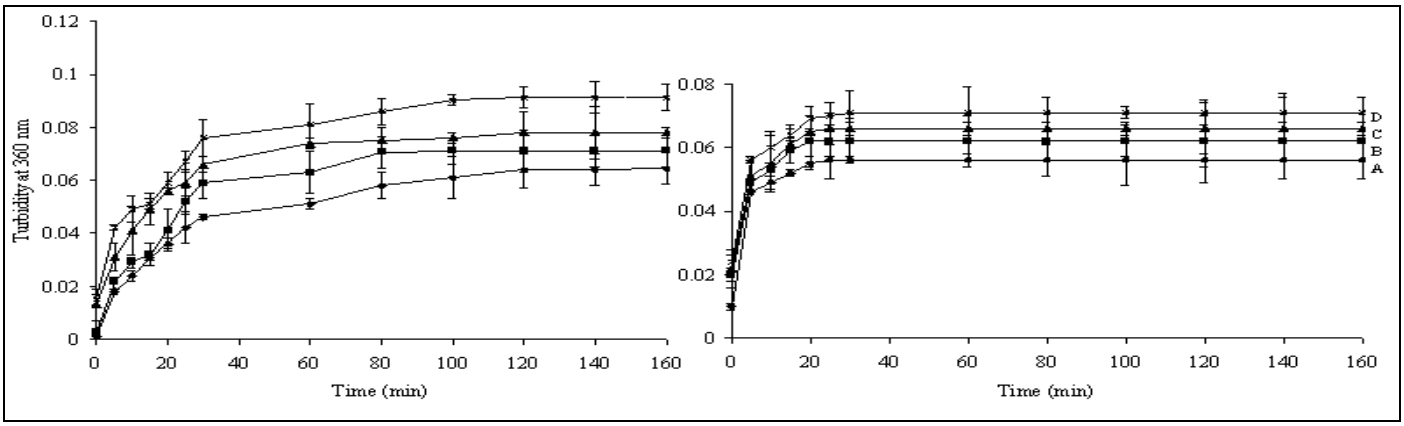
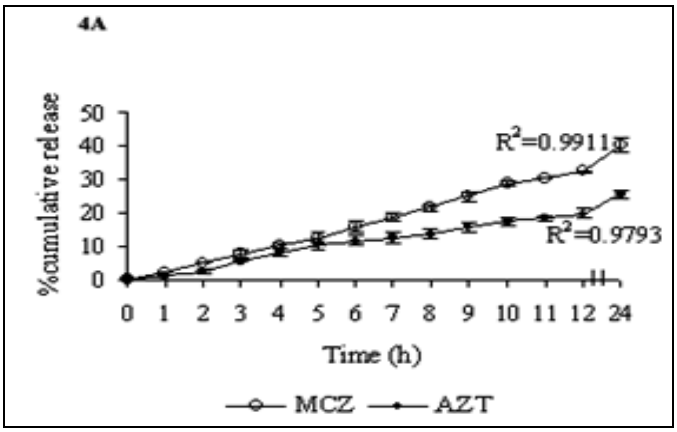


FIG. 3: AGGREGATION OF (A) DOUBLE LIPOSOMES WITH INNER GALACTOSYLATED LIPOSOMES; (B) INNER GALACTOSYLATED LIPOSOMES ALONE, BY *RICINUS COMMUNIS* AGGLUTININ. AT 25 °C VARYING AMOUNTS OF LECTIN WERE ADDED TO GALACTOSYLATED LIPOSOMES

A (•), B (■) and C (▲) denote 5, 10 and 20μg/mL of lectin, respectively. D (×) denotes 30, 50 and 60μg/mL of lectin concentration (mean ± S.D. n=3)

To determine the effect of galactose coating and outer liposomal membrane on AZT release rate, both uncoated and galactosylated liposomal formulations entrapped in outer liposomal bilayer (having MCZ) were taken for drug release study. Percent cumulative drug release of AZT from inner liposomes was significantly higher (P = 0.0052) with DLMA as compared to Gal-DLMA. The regression coefficient values of % cumulative drug release in 24 h for both the liposomal formulations were very close to one suggesting the zero order release profile. Percent cumulative AZT release in 24 h was significantly (P<0.001) low indicating prolonged release carrier potential of double liposomes with inner galactosylated liposomes as compared to that of double liposomes with inner uncoated liposomes (**Fig. 4. A-B**). Insignificant (P>0.05) difference in the release of MCZ between the two liposomal formulations was observed. Percent cumulative drug release of AZT from uncoated liposomal formulation and galactosylated liposomal formulation, both without outer liposomal bilayer, was significantly higher (P<0.001, data not shown) as compared to the same formulation but entrapped in outer liposomal membrane.



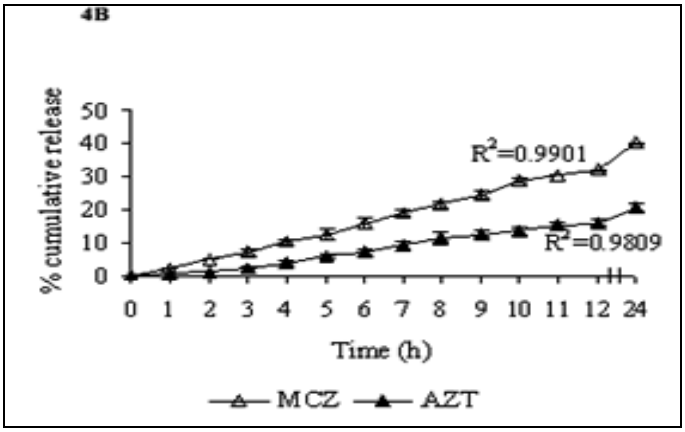


FIG. 4: IN VITRO DRUG RELEASE OF AZT (●) AND MCZ (○) FROM DOUBLE LIPOSOMES (A) WITH UNCOATED INNER LIPOSOMES AND, (B) WITH INNER GALACTOSYLATED LIPOSOMES AT 37°C IN PBS (pH 7.4)

A break (‖) indicates the change from linearity (means ± S.D. n=3)

Cellular Drug Uptake Study: From table 3 it is observed that drug uptake by mononuclear cells was higher from liposomes. AZT and MCZ uptake was significant (P<0.001) after their encapsulation in liposomes as compared to free drug uptake. Maximum AZT uptake (7.9 fold enhancement) was observed when double bilayer galactosylated liposomes were used; whereas, in case of double bilayer uncoated liposomes it was 3.8 fold increase compared to the free drug. Similarly, MCZ uptake was 4.7 times greater after encapsulation in liposomes irrespective of inner liposome coating as compared to free drug.

Insignificant (P>0.05) MCZ uptake was observed between double bilayer galactosylated liposomes and double bilayer uncoated liposomes, which may be due to the fact that MCZ was present in the outer liposomes. The inner galactose coated liposomes did not interfere with the release of MCZ from outer liposomes. In case cell suspension incubated with drug unloaded double liposomes with galactosylated inner liposomes the uptake of AZT and MCZ after 48 h from Gal-DLMA was insignificant (P>0.05) as compared to that from DLMA indicating the saturation of galactose specific receptors present on the mononuclear cells.

TABLE 3: AZT AND MCZ UPTAKE BY MONONUCLEAR CELLS AT DIFFERENT TIME POINTS AT 37°C. INHIBITION EFFECT SHOWS THE RESPECTIVE DRUG UPTAKE FROM Gal-DLMA AFTER THE EXCESS AMOUNTS OF DRUG UNLOADED DOUBLE LIPOSOMES WITH GALACTOSYLATED INNER LIPOSOMES HAVE BEEN ADDED TO THE CELL SUSPENSION

Time (h)	Drug uptake (µg/million cells)*							
	AZT				MCZ			
	free MCZ+AZT	DLMA	Gal-DLMA	Inhibition effect	free MCZ+AZT	DLMA	Gal-DLMA	Inhibition effect
0	0	0	0	0	0	0	0	0
1	0.011±0.001	0.180±0.062	0.190±0.012	0.170±0.014	0.009±0.001	0.160±0.020	0.165±0.010	0.160±0.010
4	0.056±0.002	0.390±0.024	0.590±0.020	0.360±0.025	0.046±0.001	0.520±0.009	0.520±0.020	0.500±0.030
8	0.081±0.011	0.65±0.016	0.910±0.010	0.640±0.124	0.078±0.041	0.720±0.014	0.830±0.015	0.690±0.020
20	0.102±0.014	0.814±0.032	1.201±0.010	0.795±0.114	0.096±0.011	0.856±0.021	0.870±0.011	0.870±0.015
48	0.235±0.050	0.912±0.098	1.865±0.085	0.904±0.107	0.199±0.045	0.948±0.230	0.947±0.150	0.956±0.16

DLMA: Double liposomal formulation with AZT in non-galactosylated inner liposomes and Miconazole nitrate in the bilayer of outer liposomes.
Gal-DLMA: Double liposomal formulation with AZT in the galactosylated inner liposomes and Miconazole nitrate in the bilayer of outer liposomes.
*All values are expressed as mean ± S.D. (n = 6).

***In Vivo* Antifungal Activity:** Treatment with free MCZ solution alone and when given in combination with AZT showed significant (P < 0.05) reduction in CFU values in the lungs only when compared with non-treated control rats. Both drugs encapsulated in double liposomes were able to reduce CFU values to a significant (P<0.05) extent in lungs, liver, kidney, spleen, pancreas and plasma as compared to free MCZ solution (**Fig. 5**). AZT alone was not able to reduce CFU. The survival rate of rats increases where miconazole is entrapped in double liposomes.

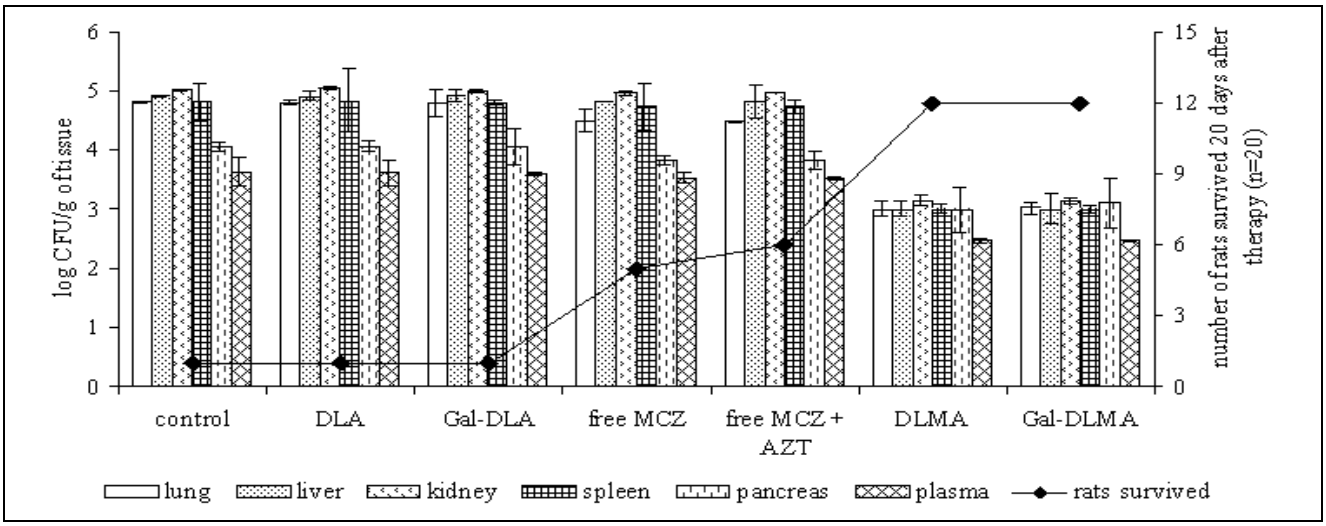


FIG. 5: COLONY-FORMING UNITS (CFU) OF *CANDIDA ALBICANS* IN DIFFERENT ORGANS OF RATS (n = 6) AND NUMBER OF SURVIVAL OF INFECTED RATS (n = 20) AFTER ADMINISTRATION OF DIFFERENT FORMULATIONS. Gal-DLA AND DLA REPRESENT DOUBLE LIPOSOMAL FORMULATION WITH INNER AZT GALACTOSYLATED LIPOSOMES AND WITH AZT INNER UNCOATED LIPOSOMES, RESPECTIVELY. (MEAN ± S.D.)

Plasma Distribution and Hepatic Localization: The incorporation of AZT and MCZ in liposomal vesicles strongly altered their distribution pattern. After DLMA injection, AZT plasma levels monitored were more than 0.86±0.01ng/mL upto 2 h post-injection. In contrast with DLMA, plasma AZT concentration following free AZT+MCZ injection decreased rapidly (0.01±0.001ng/mL at 2 h post-injection).

At 2 h post-injection plasma level of AZT from Gal-DLMA was less (0.81±0.001) as compared to that from DLMA (0.86±0.01). However, Gal-DLMA retained significantly larger (P<0.0005) concentration of AZT even after 12 h as compared to DLMA (**Fig. 6A**). This may be due to the fact that as galactosylated liposomes are released from double liposomes the hepatic cells take them up. This uptake is a saturable process. No further uptake occurs until the receptor becomes free. Therefore galactosylated liposomes remain in the plasma and account for the higher plasma level at 24 h post-injection. Gal-DLMA maintained a significantly higher level of AZT (P<0.001) in liver up to 24 h (more than plasma concentration) compared to free AZT+MCZ and DLMA (**Fig. 6C**). The results correlated well with studies of Banerjee *et al.,* (1994) for mannosylated liposomes [19]. Galactosylated liposomal encapsulated AZT remains in the body for longer period of time and gets distributed to various tissues (having galactose receptors) of the body.

Similarly, DLMA injection provided MCZ plasma levels ≥9.40±0.28ng/mL upto 24 h post-injection. Whereas, plasma MCZ concentration following free AZT+MCZ injection decreased rapidly

(2.11±0.14ng/mL at 24 h post-injection (**Fig. 6B**). Significant (P<0.0005) liver concentration of MCZ as compared to free AZT+MCZ was observed in case when DLMA was administered (**Fig. 6D**). Gal-DLMA showed insignificant (P>0.05) MCZ plasma and liver concentration at 72 h post-injection as compared to that of DLMA. This may be attributed to the presence of MCZ in the outer liposomal bilayer of double liposomes.

AUC and MRT in plasma after free AZT+MCZ, DLMA and Gal-DLMA are given in **table 4**. AUC of MCZ and AZT after administration of DLMA were 1.7 and 21.5 times higher, respectively whereas in case of Gal-DLMA AUC of AZT was 23.4 times as compared to free AZT+MCZ. MRT of MCZ and AZT after administration of DLMA was 1.6 and 14 times higher, respectively whereas in case of Gal-DLMA AUC of AZT was 14.6 times as compared to free AZT+MCZ. Insignificant (P>0.05) difference in AUC and MRT of MCZ obtained from Gal-DLMA and DLMA was observed.

Figure 6E shows hepatic cellular localization of AZT formulations at 24 h post injection. Significant (P<0.0005) amount of AZT was recovered from PC in case of Gal-DLMA administration as compared to NPC. AZT uptake by PC from Gal-DLMA was significant as that by free AZT+MCZ and DLMA. Insignificant (P>0.05) drug concentration was seen in PC as compared to NPC after free AZT+MCZ and DLMA administration. Uptake by PC was more because of the abundance of asialoglycoprotein receptors on them.

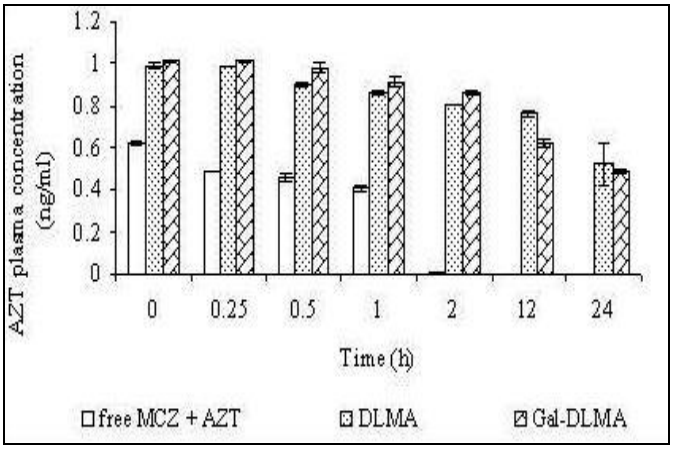
TABLE 4: AREA UNDER PLASMA CONCENTRATION TIME CURVE (AUC) AND MEAN RESIDENCE TIME (MRT) IN PLASMA AFTER INTRAVENOUS INJECTION OF DIFFERENT FORMULATIONS IN RATS

Formulation	AUC (ng.h/mL)		MRT (h)	
	MCZ	AZT	MCZ	AZT
Free AZT+MCZ	194.21 ± 4.92	0.74 ± 0.01	20.57 ± 1.14	0.73 ± 0.05
DLMA	324.25 ± 9.35***	15.93 ± 0.65***	33.21 ± 1.47***	10.22 ± 0.24***
Gal-DLMA	320.19 ± 6.56***	17.29 ± 0.12***	32.57 ± 2.18***	10.70 ± 0.02***

DLMA: Double liposomal formulation with AZT in non-galactosylated inner liposomes and Miconazole nitrate in the bilayer of outer liposomes

Gal-DLMA: Double liposomal formulation with AZT in the galactosylated inner liposomes and Miconazole nitrate in the bilayer of outer liposomes

All values are expressed as mean ± S.D. (n = 3). ***P < 0.001 (significant) as compared to free AZT+ MCZ



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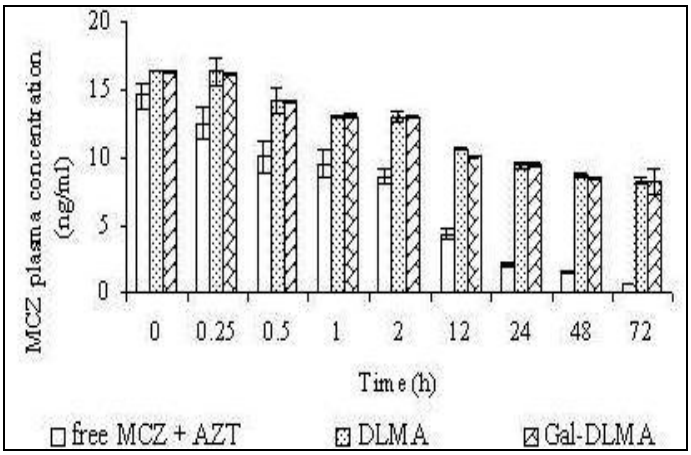

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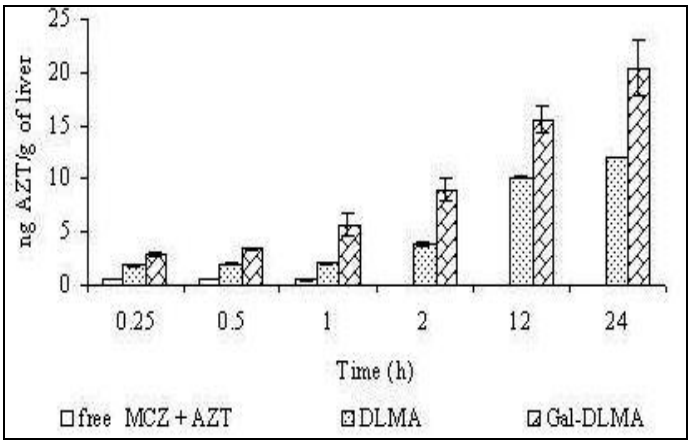

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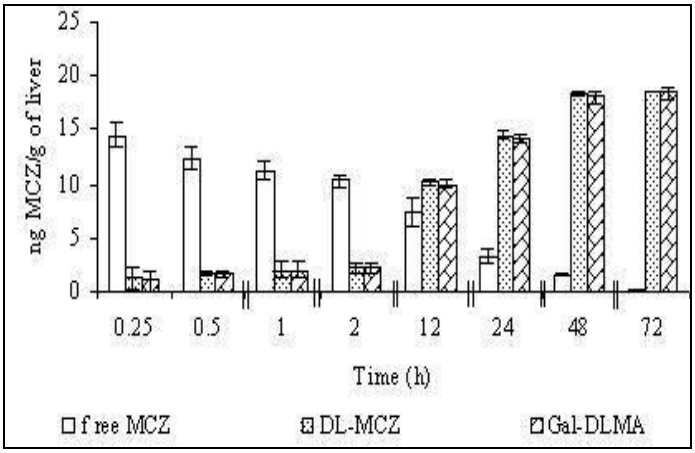

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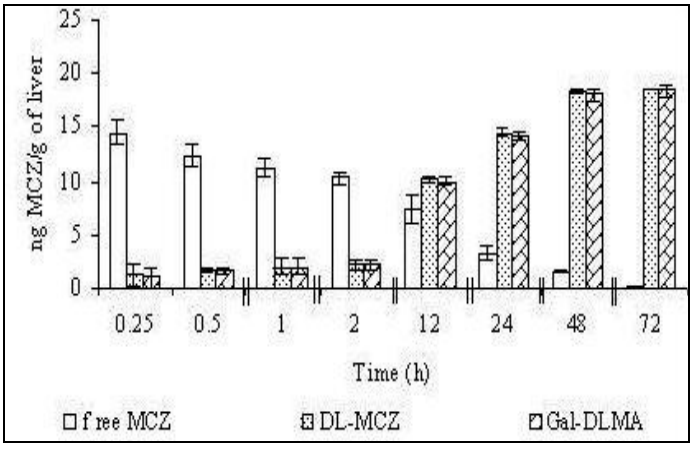

6 (E)

FIG. 6: PLASMA CONCENTRATION-TIME CURVE OF DIFFERENT FORMULATIONS (A) AZT (B) MCZ; CONCENTRATION OF (C) AZT AND (D) MCZ IN LIVER; (E) HEPATIC CELLULAR UPTAKE AT 24 H POST-INJECTION IN RATS

A break (‖) indicates the change from linearity (means ± S.D. n=3)

DISCUSSION: Hepatocytes are interesting as target cells for several reasons. The asialoglycoprotein receptor they express is well characterized and abundant, and its interaction with a ligand leads to endocytosis. Furthermore, the liver is the center organ of several genetic, acquired, and viral diseases [20].

Galactose being hydrophilic in nature cannot be used to coat liposomes. Therefore it was necessary to chemically modify galactose by conjugating it to a hydrophobic group, which allows the galactose residue to interligate with the liposomal membrane. Synthetic glycolipid, which could be incorporated into liposomes, has been prepared by chemical coupling between galactose derivatives and phosphatidyl ethanolamine by amide bond formation.

Liposomes targeting to specific cells has been achieved with natural and synthetic lipids by introducing glucidic ligands onto the surface of the vesicles. We have prepared glycosylated liposomes using synthesized galactolipid (Gal-PE). Thioether linkage was used, as it was reported to be chemically more stable, resistant to the action of glycosidases and not detrimental for the receptor affinity [16]. Elongation of a spacer arm using thiopropionic acid may have a highly beneficial effect on the ability of glycosylated liposomes to form a strong complex with protein receptor [10].

The optimized formulation of inner lipoomes shows an average particle size of 130.45±1.24 nm while that of the outer liposomes shows an average particle size of 1.20±0.01 μm. The inner liposomes have galactose coating on its surface, which makes them reside in the aqueous interior of the outer liposomes. There are ample examples of nanoparticles and liposomes of size range of 200 nm entrapped within liposomes [21]. Unaltered T_m (transition temperature) value of both liposomes indicates that AZT and MCZ did not

interfere with any of the constituent of double liposomes. Increase in turbidity in case of double liposomal formulations (with inner Gal-PE liposomes) may be due to the galactose specific nature of *Ricinus communis*. This lectin agglutination represents an in vitro model for assessing the affinity of galactosylated liposomes for asialoglycoprotein receptors.

Drug release data projects that double liposomes having inner liposomes, either uncoated or galactosylated, have sustaining release potential. Percent cumulative release of AZT and MCZ in 24 h from double liposomes with uncoated inner liposomes was found to be 25.4±1.2 and 40.2±2.1, respectively. Gal-DLMA showed 20.6±0.9 and 40.1±0.36 % cumulative AZT and MCZ, respectively. This confirms sustaining release potential of double liposomes. Bilayer of outer liposomes poses a barrier for the release of inner liposomal formulation. This may be the reason for more sustained release of AZT. Significantly ($P<0.05$) less (20.6±0.9) sustained release of AZT in case of galactosylated liposomal formulation with outer bilayer may be attributed to the presence of protective coat of galactose on liposomal surface, which interferes with the drug release.

The release studies showed upto 20-40% release of encapsulated drugs from the liposomes within 24 h while the plasma level remained much higher upto 72 h since the PEGylated outer liposomes releases the drug at a sustained rate upto 72 h. The drug release profile of these double liposomes upto 72 h has been added in Figure 4 A-B. Moreover, the drug has got a biological half-life of about 24 h and an abnormally high plasma protein binding (90%), which accounts for the higher plasma concentration upto 72 h. The plasma level remained high for a long time because of the steric stabilization of PEG rendering stealth effect to these liposomes. This release potential may be beneficial in case when the drug has to be delivered to the infected cells. Galactosylated liposomes will not deliver their content to plasma. After coming in contact with asialoglycoprotein receptors on liver, galactose specific receptors on monocytes and dendritic cells; they may release AZT in the vicinity of target site.

Free MCZ and its combination with AZT were able to reduce CFU in the lung only whereas double liposome entrapped MCZ was able to reduce CFU in other tissues /organs studied (Fig. 5). This may be due to the prolonged release sustaining potential of double liposomes, which remain in the tissue for longer periods of time. No earlier report is available regarding this aspect. Encapsulation in vesicles may help in the reduction of dose as well as toxicity associated with free drug.

The extent of drug uptake from liposomal formulations is an important parameter for estimating the dosage of the formulation. PEG-lipids in the outer liposomes have been used to prevent liposomal formulation being taken up by the mononuclear phagocyte systems [22-24]. Hydration of PEG provides a water shell to the liposome surface resulting in steric stabilization [25]. Once inner galactosylated liposomes are released from double liposomes they are taken up by asialoglycoprotein receptors on the mononuclear cells.

Maximum uptake (1.865±0.085) was seen in case of galactosylated liposomes with outer bilayer (Table 3). This may be due to the presence of asialoglycoprotein receptors on the mononuclear cells. It is highly desirable that drug uptake from liposomal formulations be maximal so that a smaller dosage of the formulation would suffice to achieve an optimal therapeutic effect. Moreover, small doses are convenient to the patient in terms of parenteral administration.

By grafting different glycosides on the surface of liposomes, it is possible to direct the latter to different cell types of rat liver [26]. Galactosylated liposomes gets bound with the galactose specific receptors in the body. Galactosylated vesicles remain in the systemic circulation until and unless the binding site becomes available for them to bind. These systems do not release their content in the plasma but reside there resulting in enhanced MRT (10.22±0.24 h) and increased plasma level of AZT. Larger uptake by liver is due to the galactose particle receptor present on them. These receptors are present on T4 and other cells of the immune system, which are the main reservoirs of HIV-1.

Hepatic localization study revealed that Gal-DLMA was taken up by liver PC, due to asialoglycoprotein receptor present on these cells [27-28]. Managit *et al*., also reported that uptake of galactosylated liposomes was 7.7 fold higher than that of non-parenchymal cells [29]. Liposomal gene carriers possessing the cationic charge necessary for plasmid DNA binding and galactose residues as a targetable ligand for liver parenchymal cells have been reported [30-31]. Galactosylated liposomes were able to enhance the liver uptake of highly lipophilic drugs [32-34].

Three types of cells are representative of cellular reservoirs:

(i) Quiescent CD4+ lymphocytes (non-productive HIV-1-infected lymphocytes);

(ii) Macrophages, monocytes (M/M) and dendritic cells; and

(iii) Follicular dendritic cells [35].

The potential of lipid-based drug carriers to deliver therapeutically active agents specifically to the following four liver-associated cell populations: hepatocytes (parenchymal cells), Kupffer cells (resident liver macrophages), sinusoidal endothelial cells and intrahepatically growing colon carcinoma cells have been reported. Macrophages colonize the liver (Kupffer cells), lungs (alveolar interstitial macrophages), spleen, lymph nodes, thymus, gut, marrow, brain, connective tissue and serous cavities. Mannose receptors on the macrophage surface have been exploited for their ability to recognize and smuggle the extracellular cargo into the cell [36]. Human B and T lymphoblastoid cells express mannose, glucose, N-acetyl galactosamine, xylose and β-lactose specific lectins.

The galactosylated inner liposomes diffuse out of the outer liposomal membrane in a controlled rate and interact with asialoglyoprotein receptors expressed on parenchymal cells and monocytes. The PEGylated outer liposomes circulate within the plasma allowing the Miconazole to diffuse out gradually into the circulation. As soon as the galactosylated liposomes are released from outer liposomal bilayer they are taken up by the circulating monocytes (having galactose specific lectin receptors) upto the saturation level after which they are taken up hepatic cells (parenchymal cells and non parenchymal cells).

It can be concluded that double liposomes are good candidates for delivery of MCZ and AZT. Double liposomes are able to reduce CFU in various tissues and remain in the body for prolonged period of time. Glycotargeting exploits the highly specific interactions of endogenous lectins with carbohydrates. Liposomes are commonly cleared by the reticular endothelial system (RES), often into non-parenchymal cells. Galactosylated lipid substances allowed liver specific uptake of AZT at enhanced parenchymal: non- parenchymal selectivity ratios. Drug targeting using liposomes as carriers holds much promise, especially in drug delivery to disease sites.

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